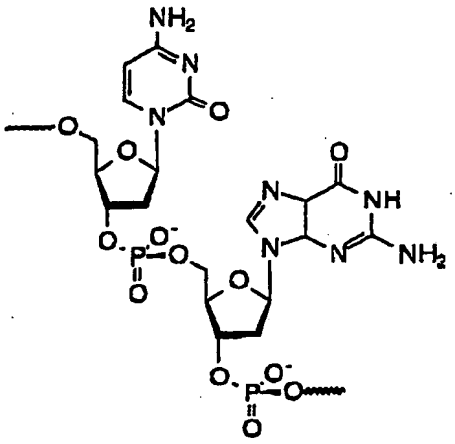


Unmethylatey CpG dinucleotide

K motif : contains essential sequences of **TCGTA** and **TCGTT**

activate monocytes / B cells, secrete IL-6

important where antibody response is essential

D ODN: containing pallindromic sequence such as **ATCGAT**
activating NK cells / secreting IFN-γ
longer sequence : e.g. GGTGCAT**CG**ATGCAGGGGGG

ODN sequence containing GA**CG**TT shows optimal response in mouse

ODN sequence containing GT**CG**TT shows optimal response in human

FIG. 1 Structure-activitity relationship of unmethylated CpG-containing nucleotide sequence

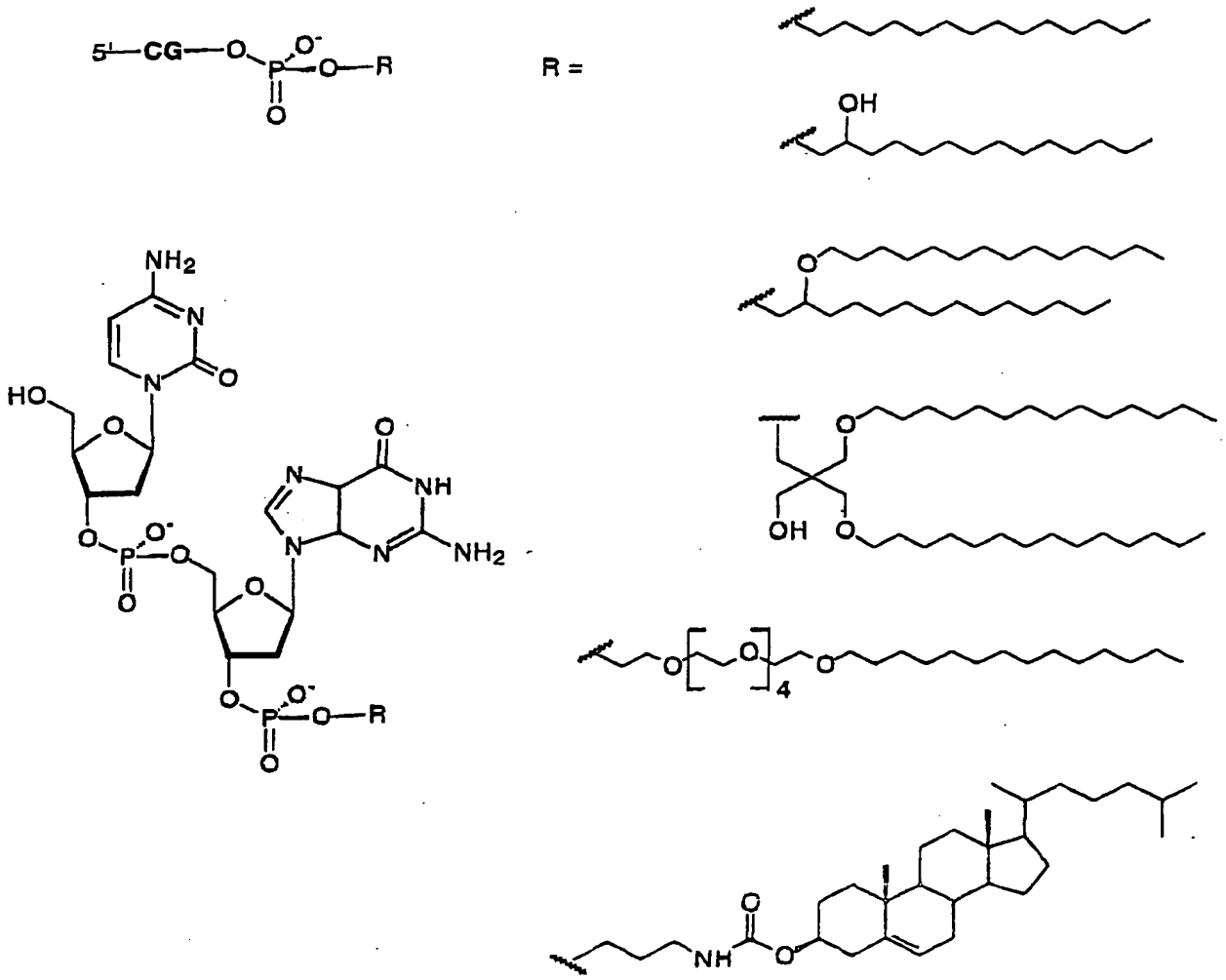


FIG. 2 CpG dinucleotide modified at 3'-end with various lipophilic group

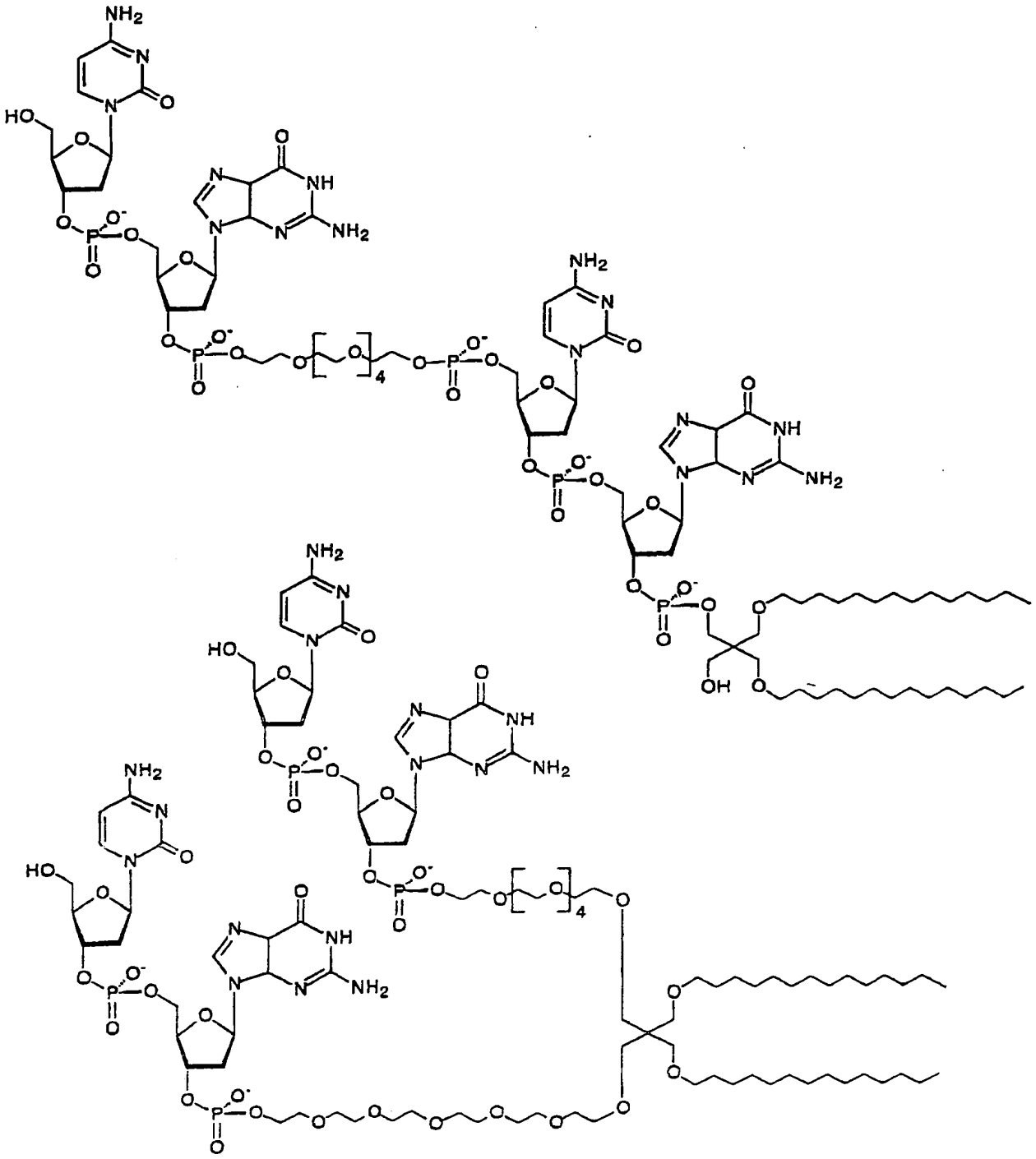


FIG. 3 Modified CpG dinucleotide as di-valent ligand

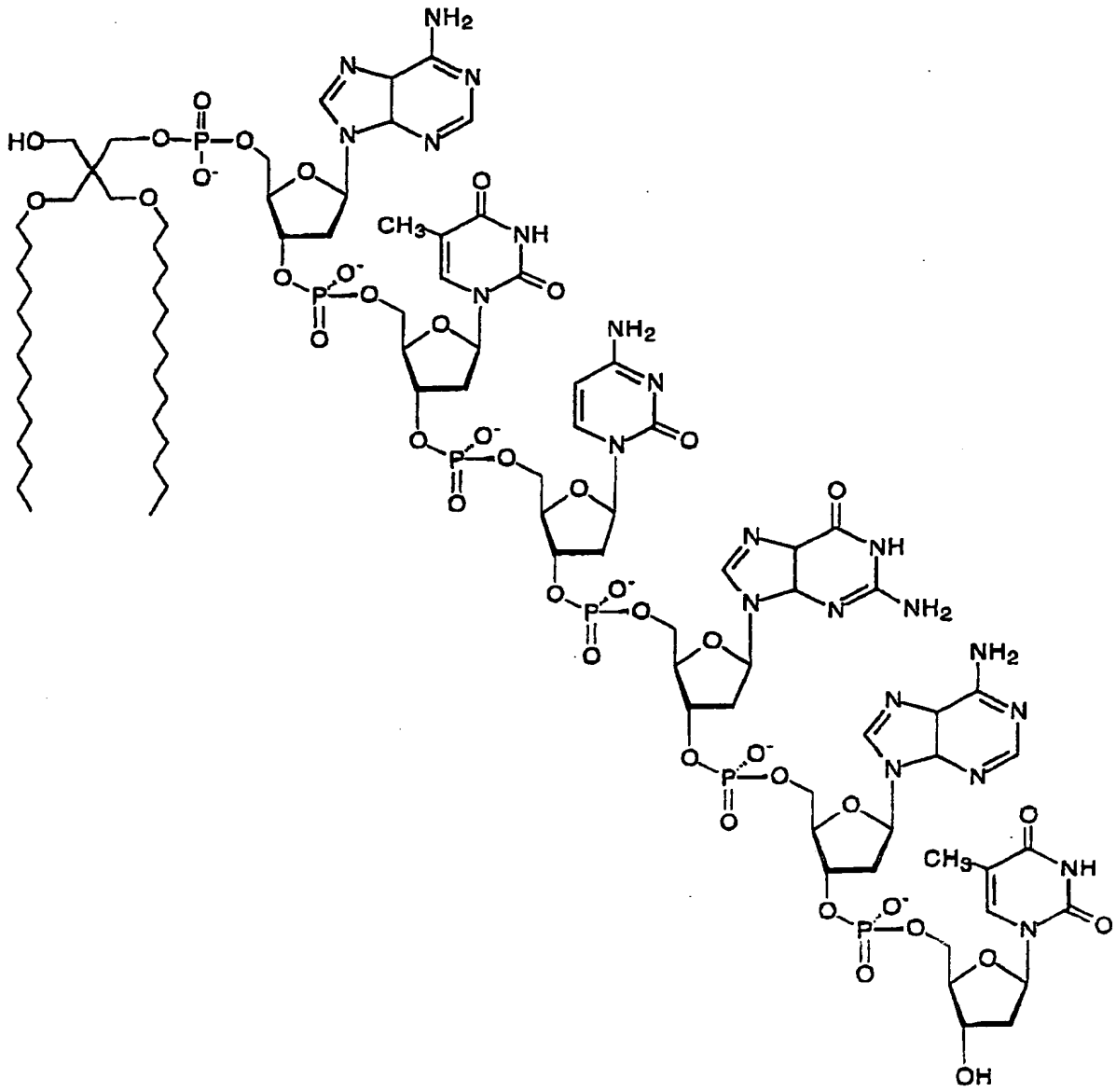


FIG. 4 Hexa-nucleotide ATCGAT modified at 5'-end with a lipophilic group

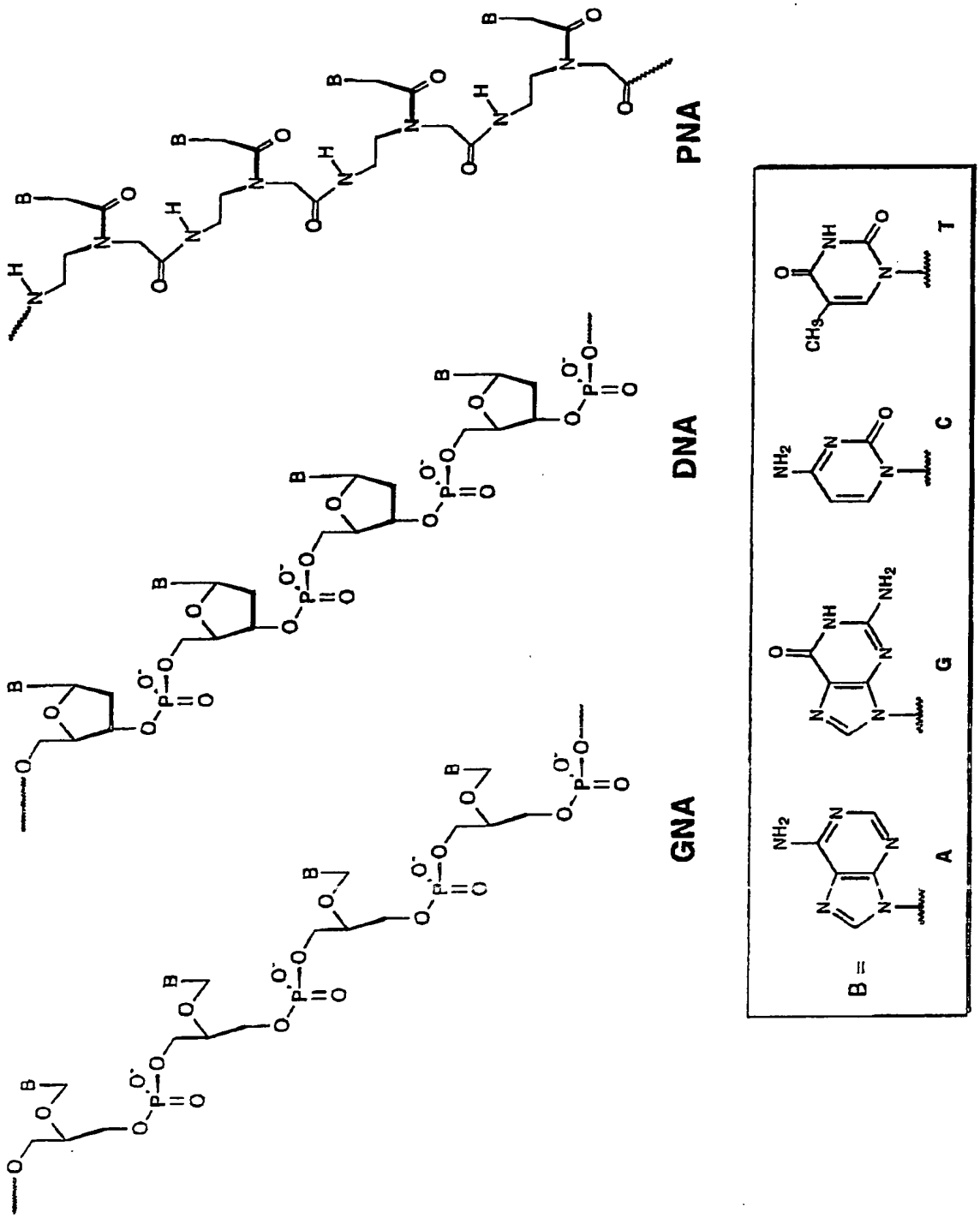


FIG. 5 Glycerol Nucleic Acid (GNA) and peptide nucleic acid (PNA) as structural mimetics of DNA

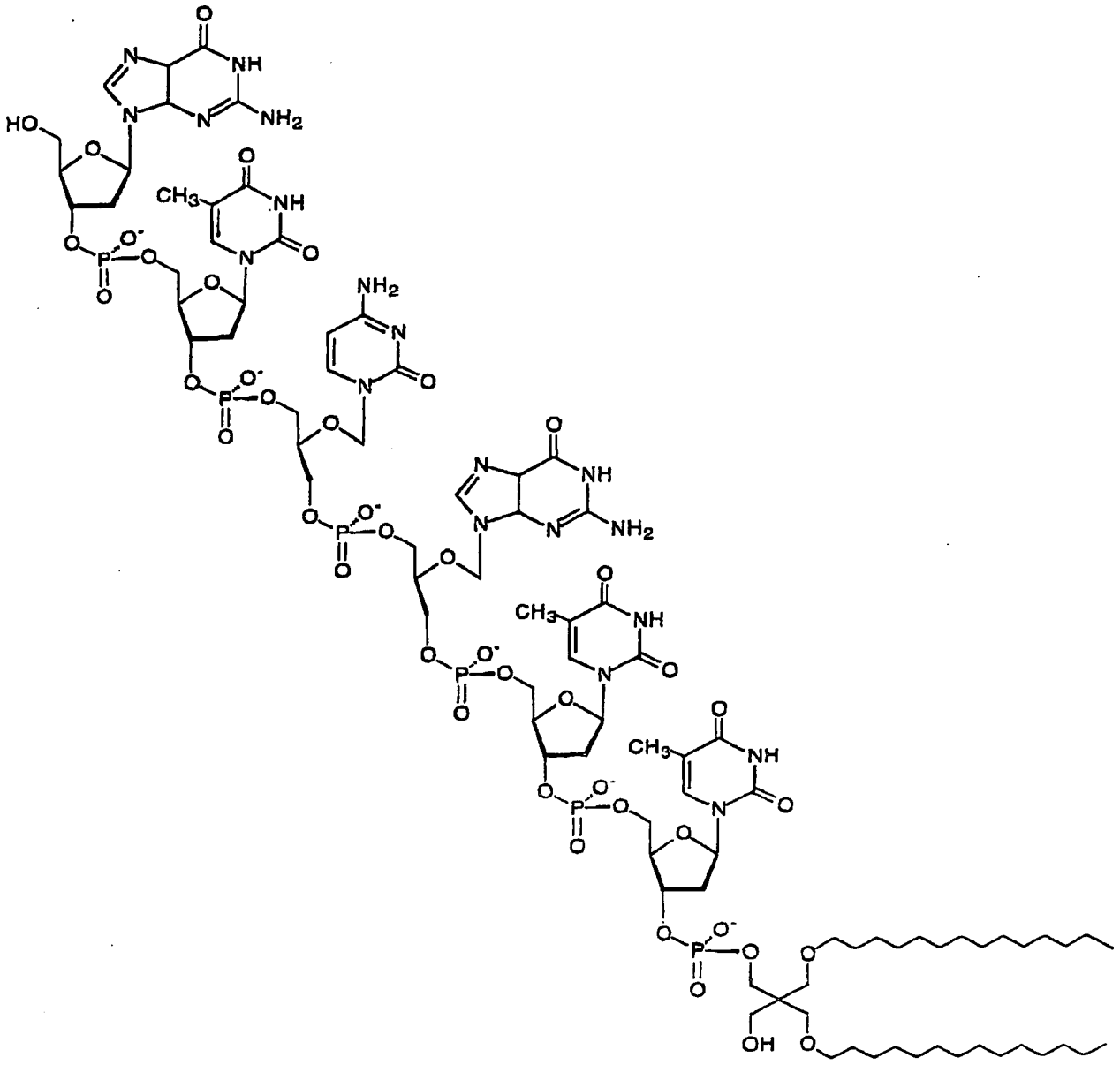


FIG. 6 Hexa-nucleotide GTcgTT modified at 3'-end with a lipophilic group, wherein cg di-nucleotide has glycerol-based backbone.

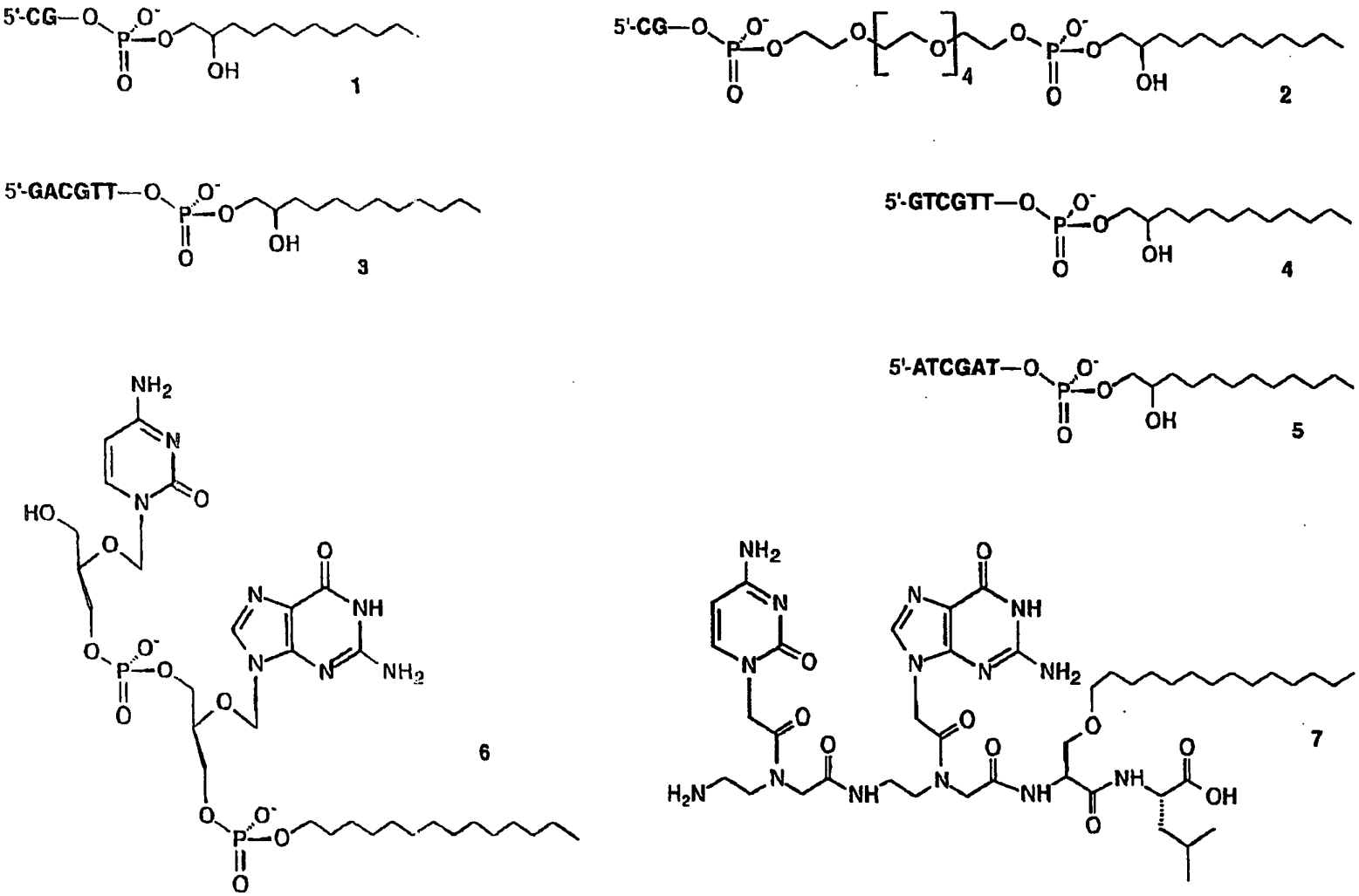
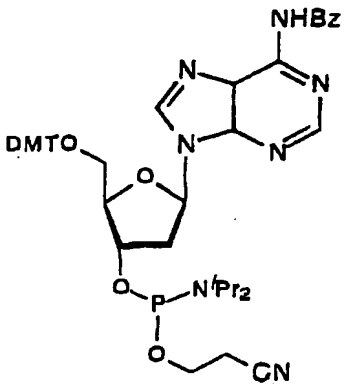
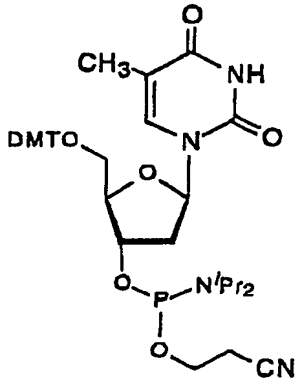


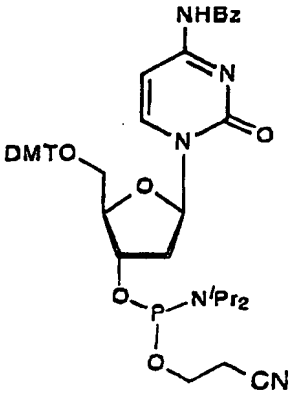
FIG. 7 Compounds prepared in this invention disclosure: CpG-containing lipidated oligonucleotides (1-5) and their mimetics based on GNA (6) and PNA (7)



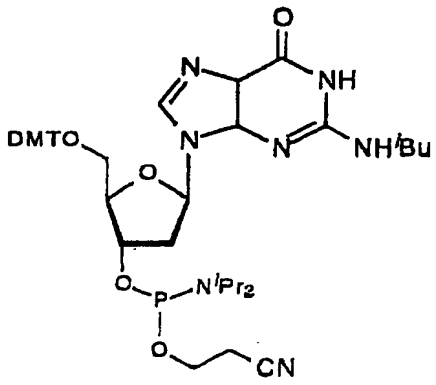
dA-CE phosphoramidite



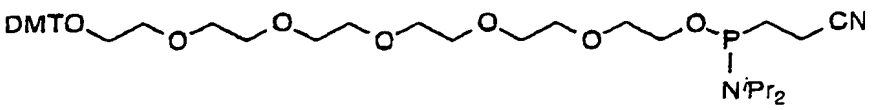
dT-CE phosphoramidite



dC-CE phosphoramidite



dG-CE phosphoramidite



Spacer-18 phosphoramidite

FIG. 8 Building blocks for solid-phase nucleotide synthesis by phosphoramidite method

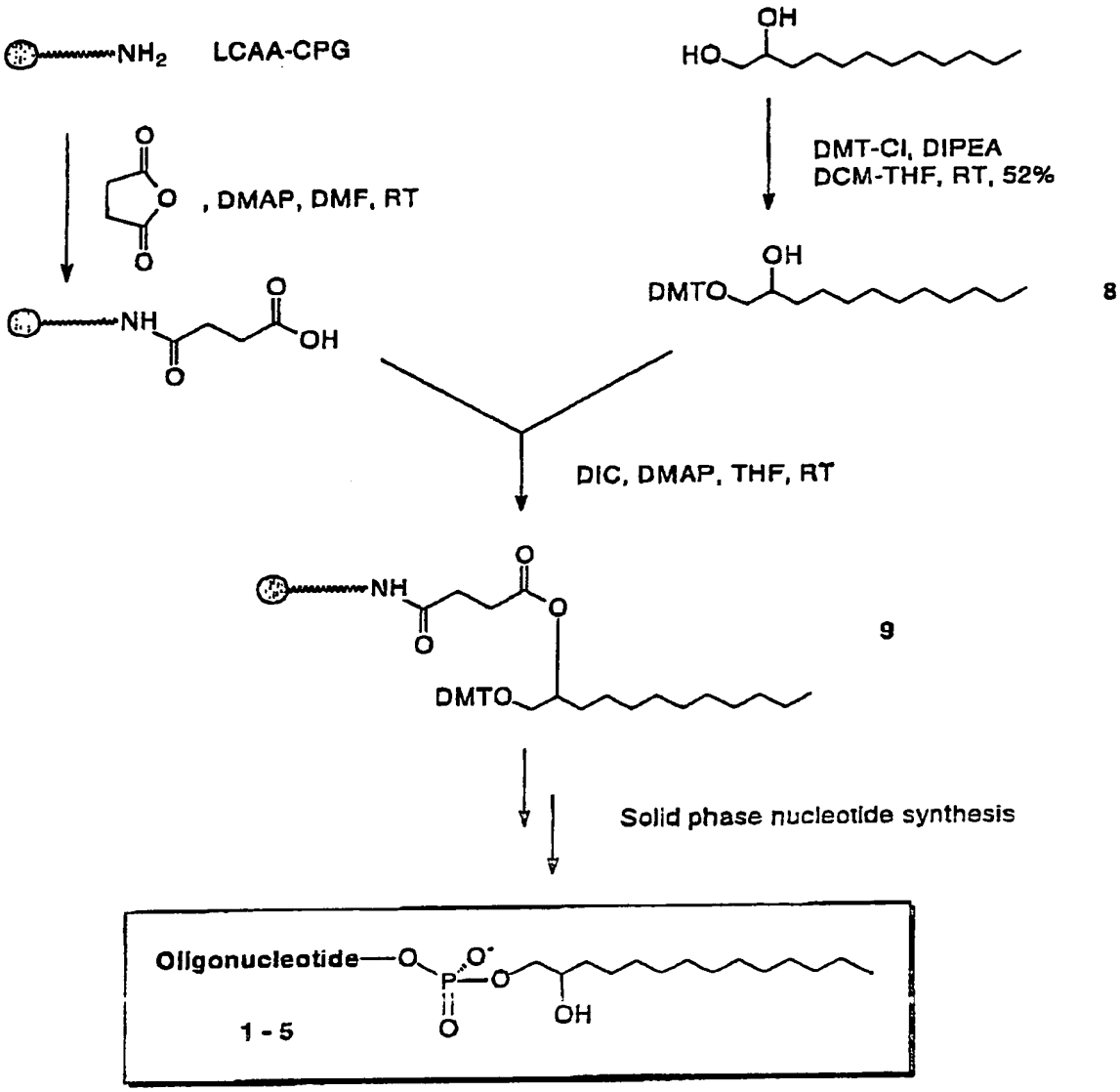


FIG. 9 Modification of lcaa-CPG (long chain aminoalkyl controlled pore glass) resin for the synthesis of lipidated oligonucleotides.

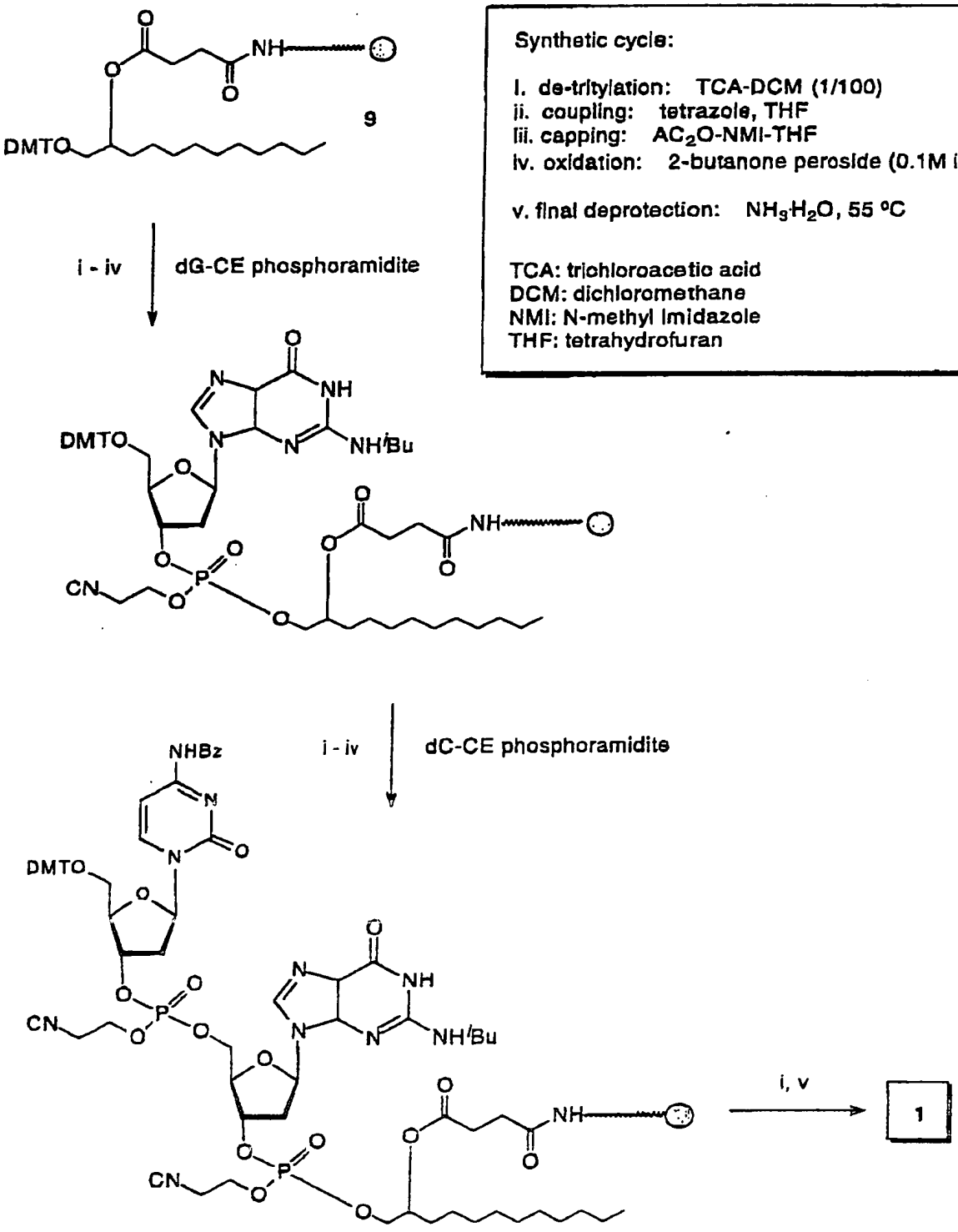


FIG. 10 Preparation of lipidated CpG dinucleotide 1 on solid phase

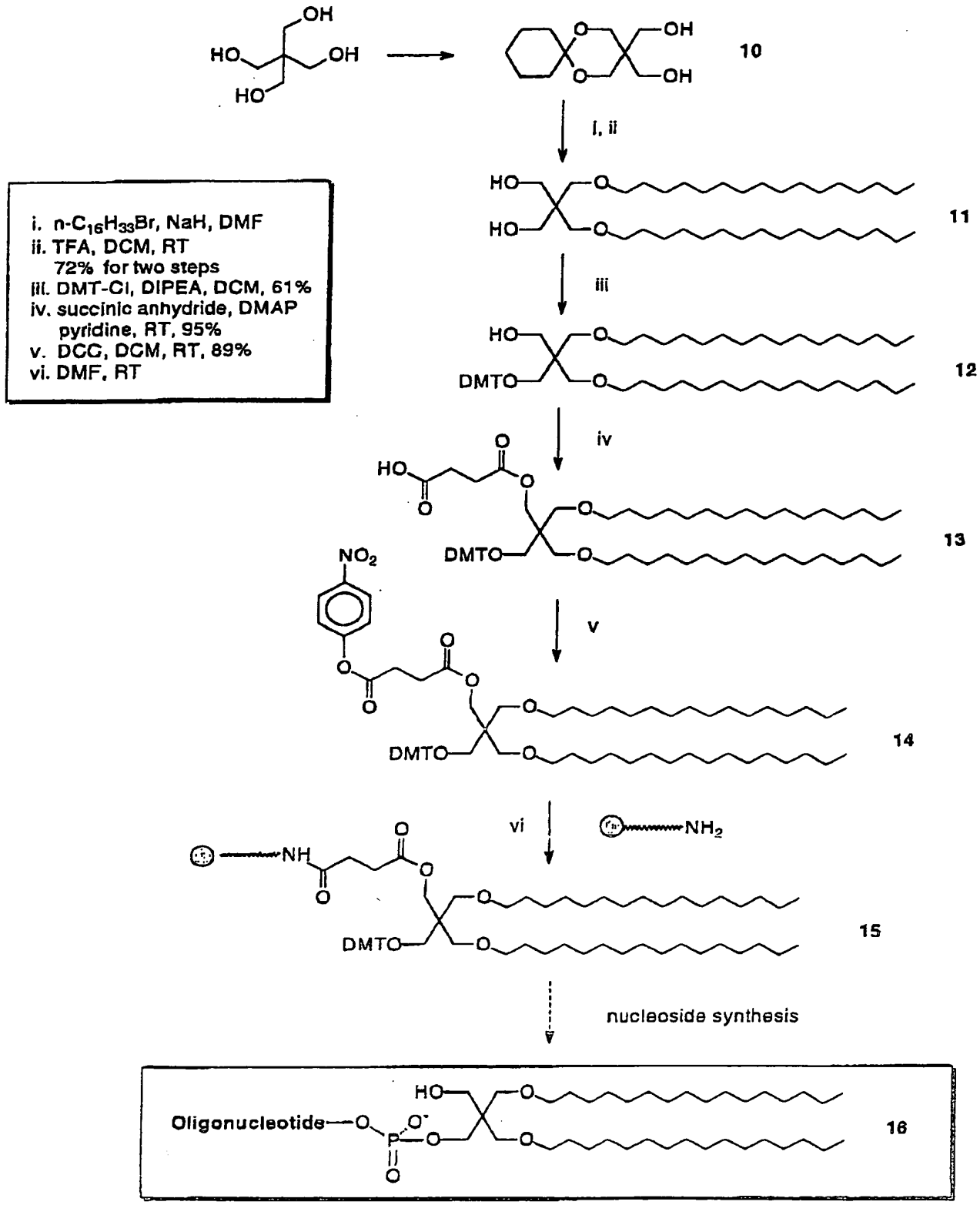
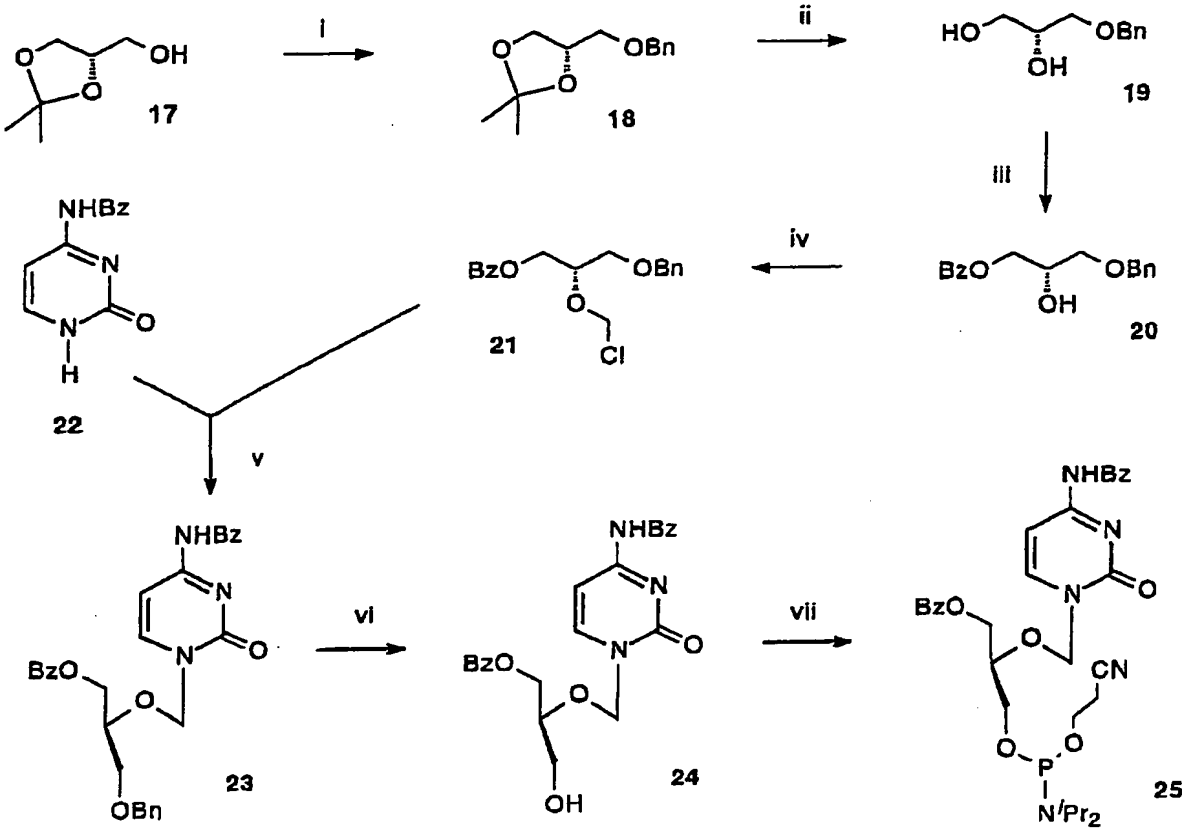


FIG. 11 Preparation of pentaerythritol-derived dilipo-alcohol 11 and its application for the synthesis of lipidated oligonucleotides (16).



i. BnBr, NaH, DMF, RT, 94%
ii. HOAc-H_2O, 40 °C, 95%
iii. BzCN, Et_3N, CH_3CN, -50 °C, 60%
iv. paraformaldehyde, DCM, HCl(g), 0 °C
v. NaH, DMF, RT, 30%; or
BSA, TBAI, DCM, RT, 35%
vi. Me_2BBr, DCM, 0°C, 76%; or
BCl_3, DCM, -78°C, 76%
vii. $(CNCH_2CH_2O)P^iPr_2$-Cl, DIPEA, DCM, RT, 79%

FIG. 12 Preparation of glycerol-cytosine phosphoramidite 25

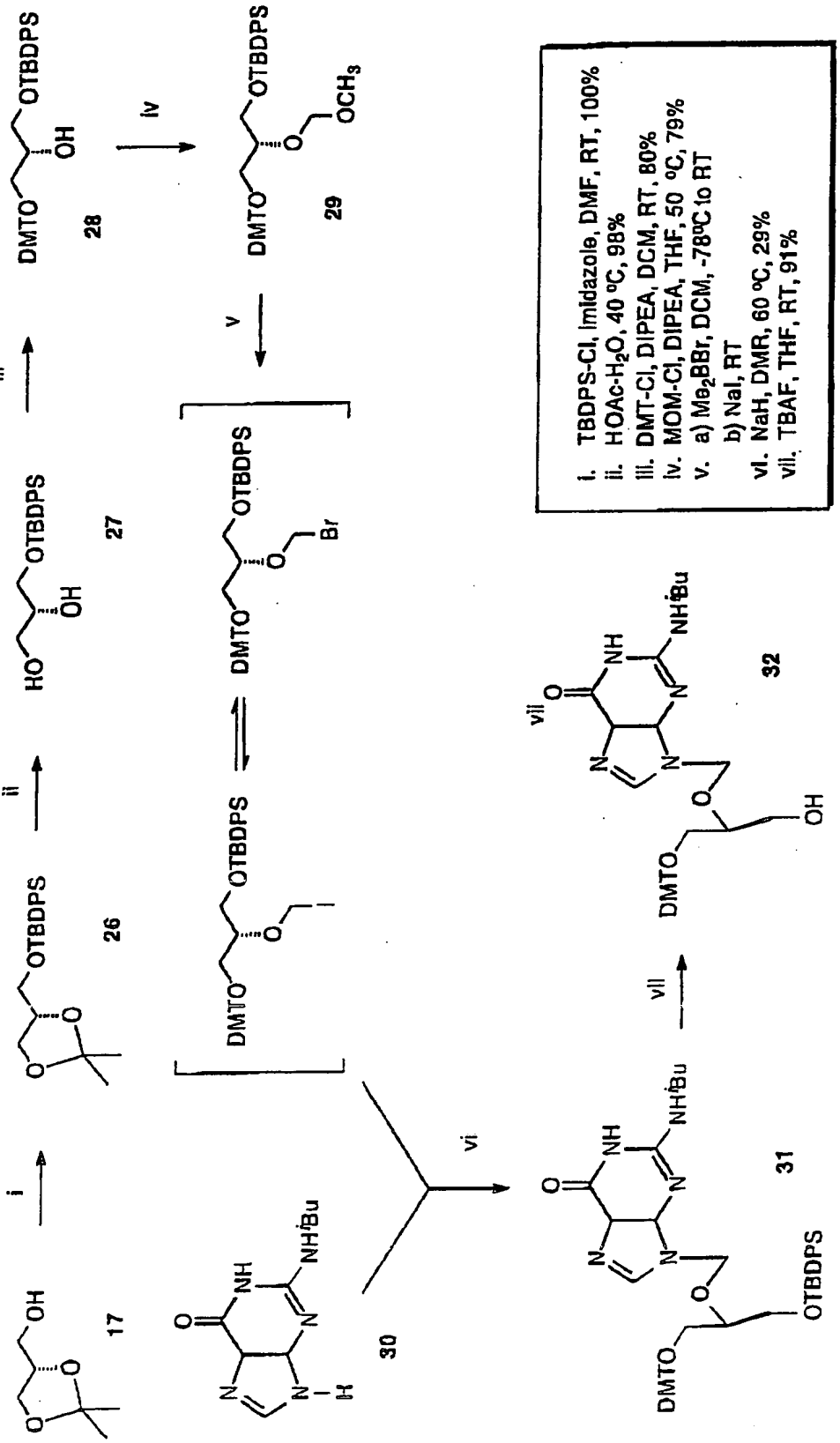


FIG. 13 Preparation of glycerol-guanosine building block 32

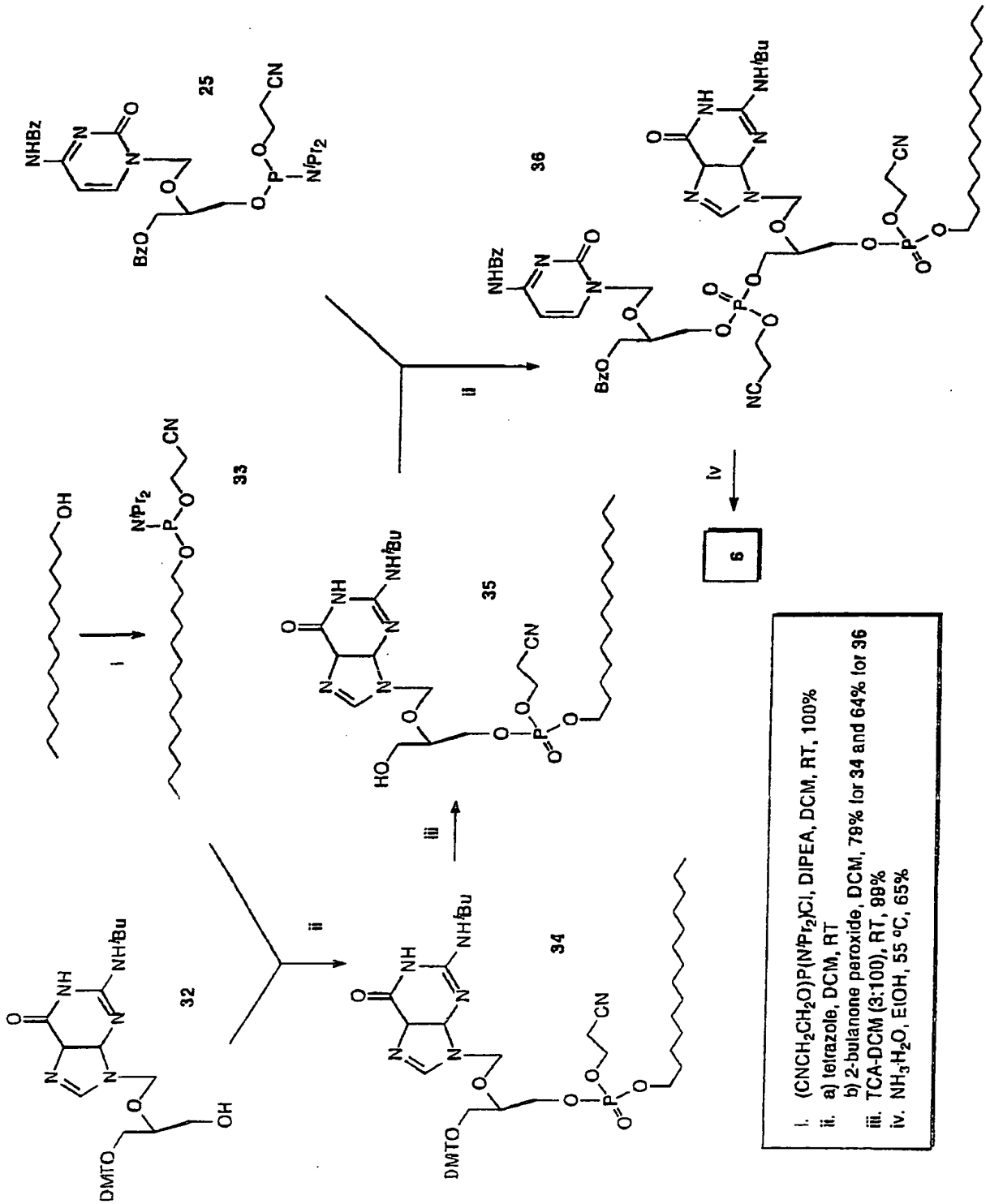


FIG. 14 Synthesis of glycerol-based CpG dinucleotide 6

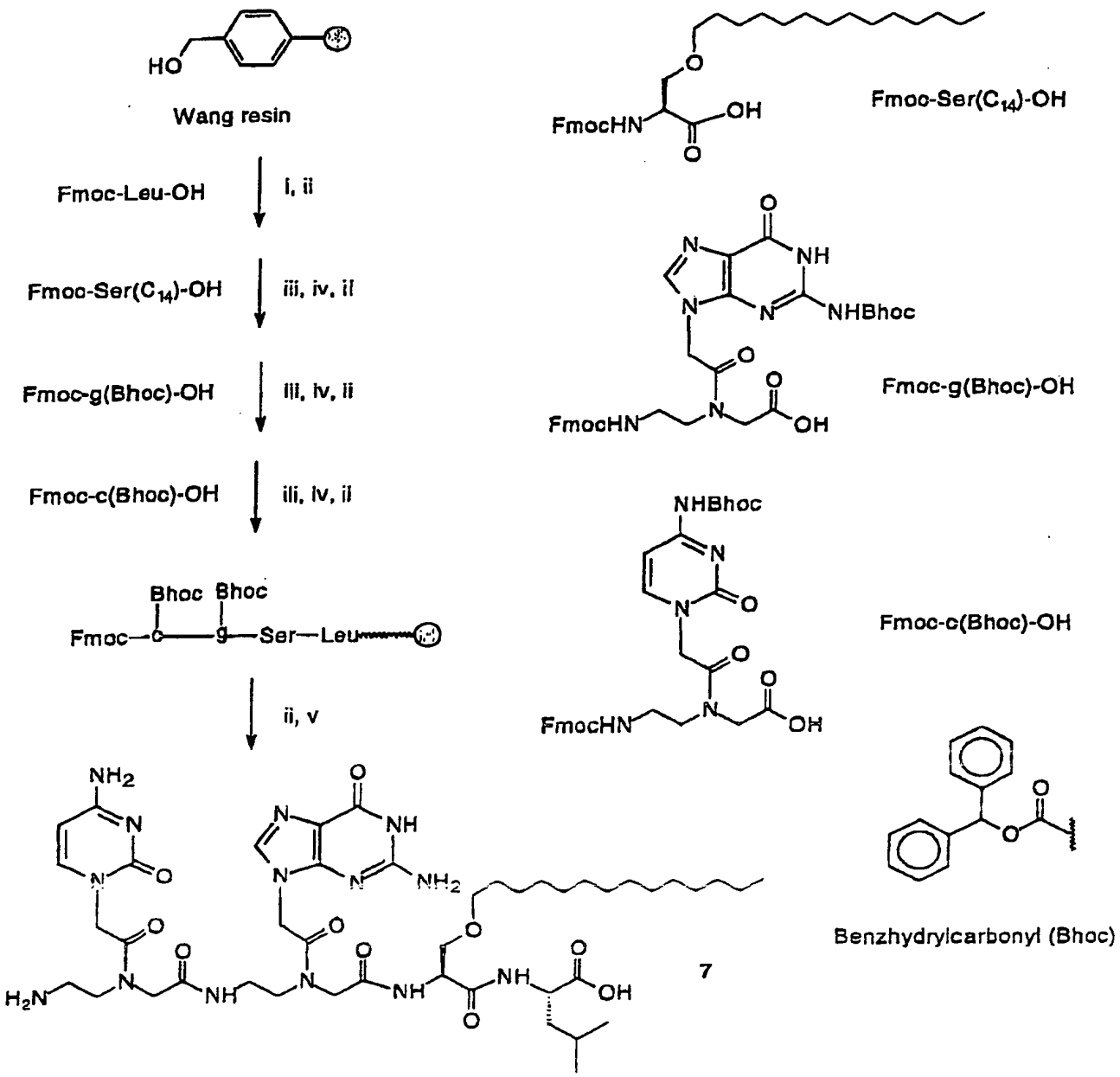


FIG. 15 Preparation of PNA-based CpG analogue 7 by standard solid phase peptide synthesis using Fmoc/Bhoc chemistry

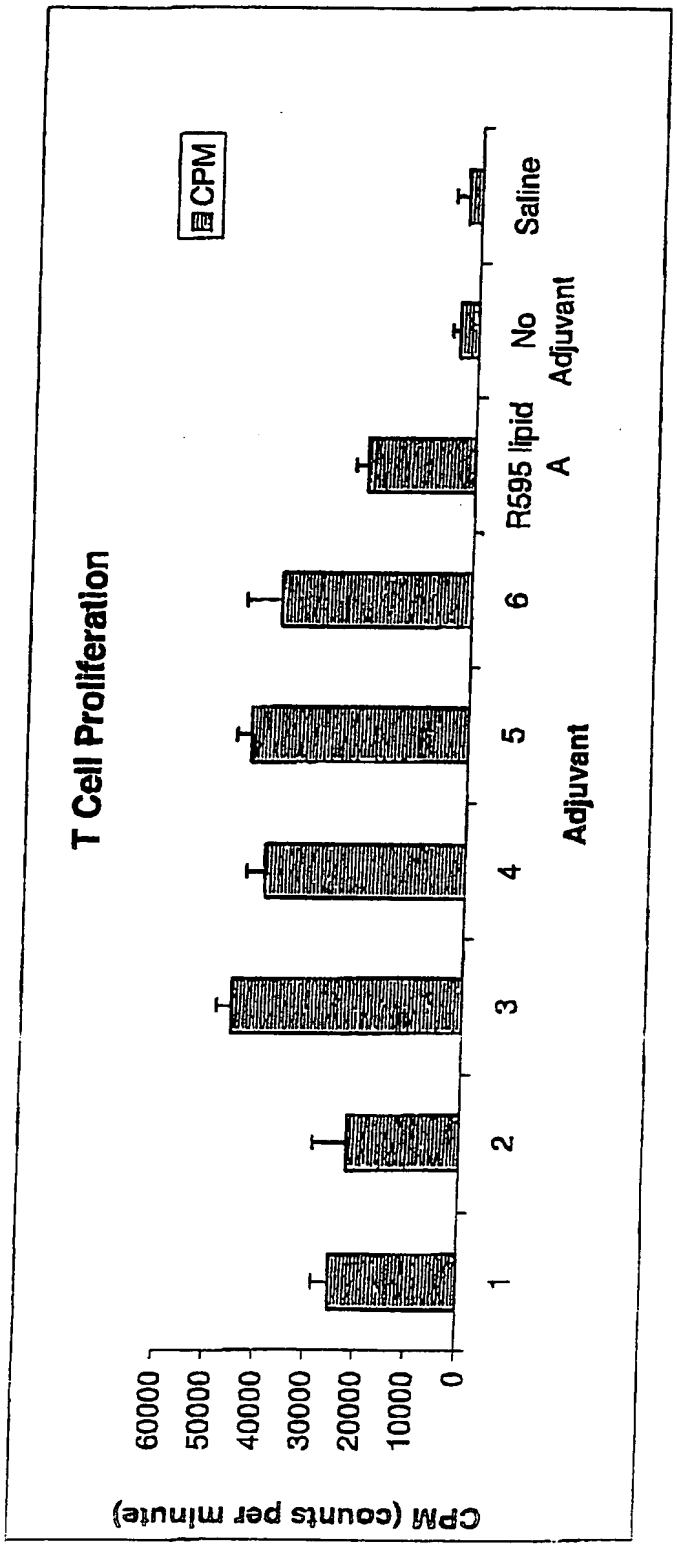
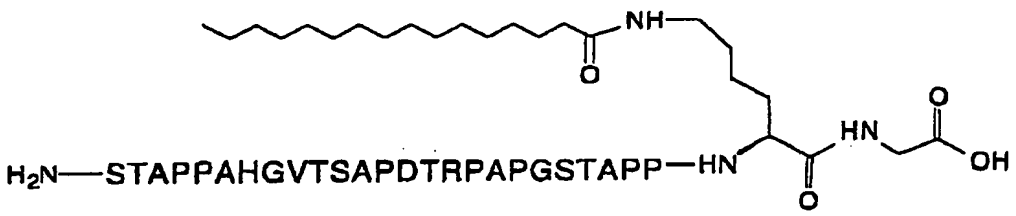


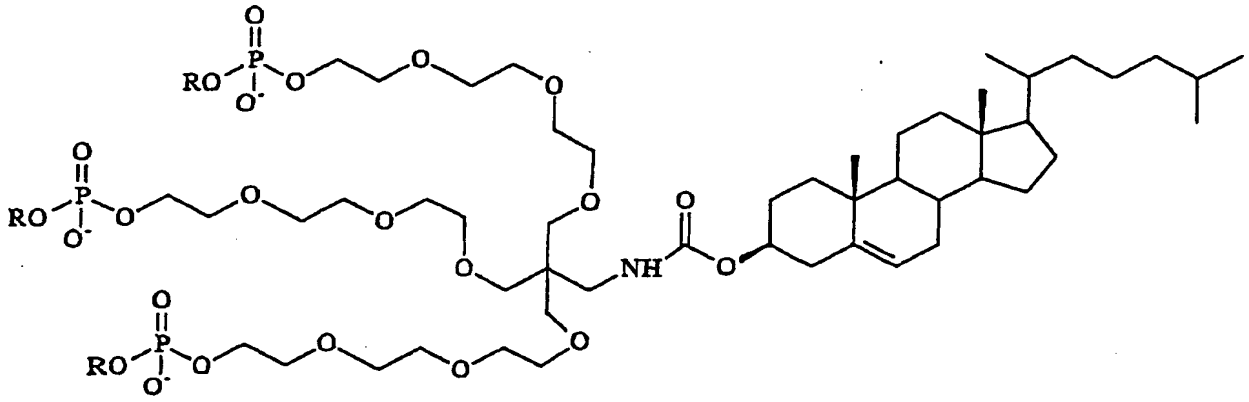
FIG. 16. Immunostimulatory adjuvant properties of CpG analogues 1–6. In vitro antigen specific proliferation of T cells from C57Bl/6 mice immunized with a single dose of BLP25 liposomal vaccine formulation. The vaccine dose contains 20 μg of MUC1-derived 25-mer lipopeptide as an antigen and 10 μg of one of synthetic CpG analogues 1–6 as an adjuvant. R595 lipid A is used for comparison, which is the natural deloxified lipid A product isolated from *Salmonella minnesota* R595 and is currently being evaluated as a vaccine adjuvant in clinic.

H_2N-STAPPAHGVTSAPPDTRPAPGSTAPPK(Pal)G-OH



BP1-148

FIG. 17 Structure of lipopeptide **BP1-148**, a modified 25-amino-acid sequence derived from tumor-associated MUC1 mucin. BP1-148 is the antigen incoporated into the liposome formulation, together with an adjuvant, e.g. compound **1 - 6**, to form the BLP25 liposomal vaccine.



R = CG, ATCGTT, GACGTT, ATCGAT, and any other ODN fragment.

Multi-valent effect is well known in many biological events. Very often the binding of a particular receptor to multi-ligands is the initial event of the multi-step activation cascade. The multi-arm of pentaerythritol (PET) provides an efficient scafold to create a multi-valent system that will function as a more efficient ligand for a specific receptor. Cholesterol-conjugated to CpG containing ODN is expected to be potential ligands for TLR-9 and thus may have immunomodulatory activity.

FIG. 18 Clustered oligonucleotide - cholesterol conjugate

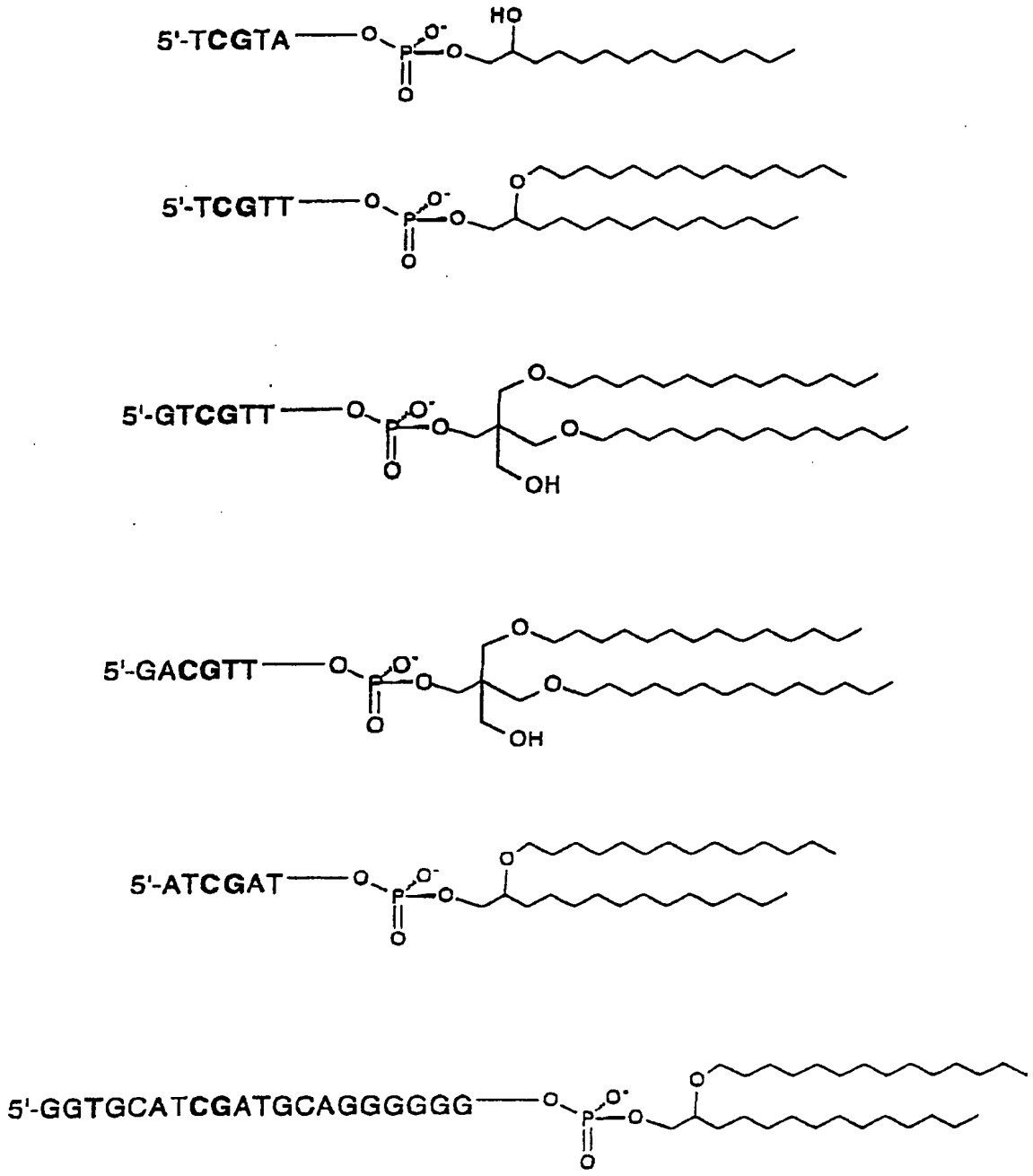
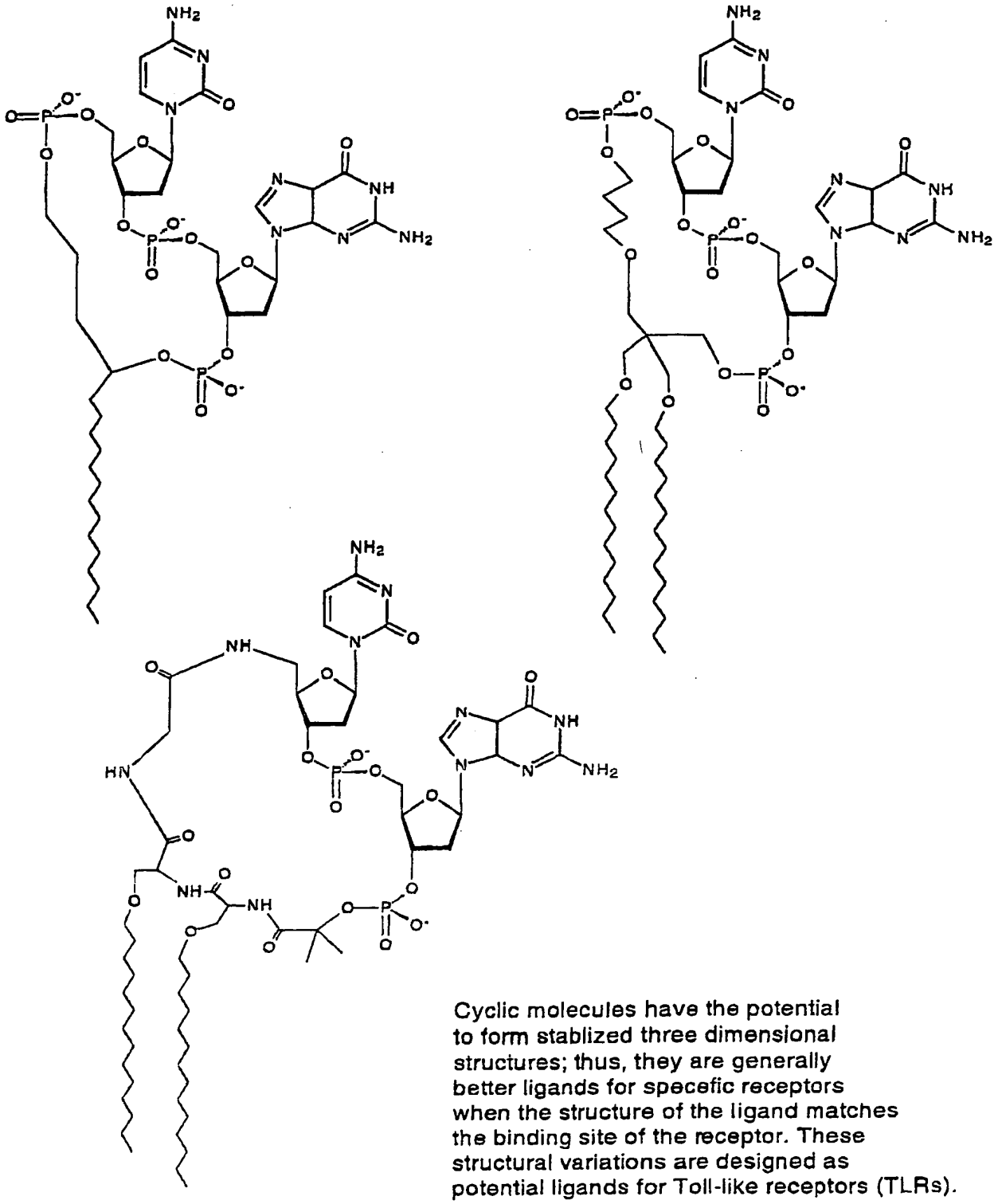
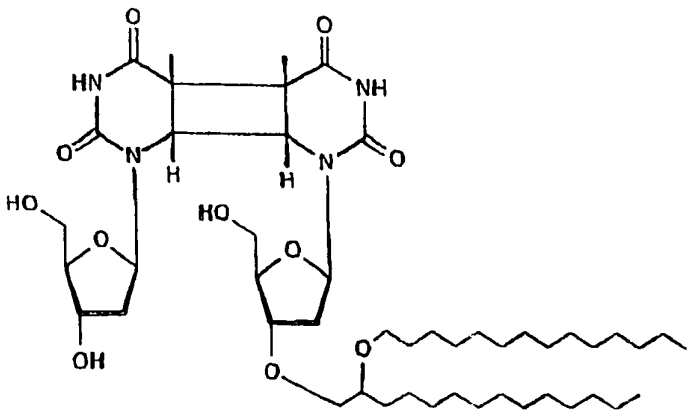


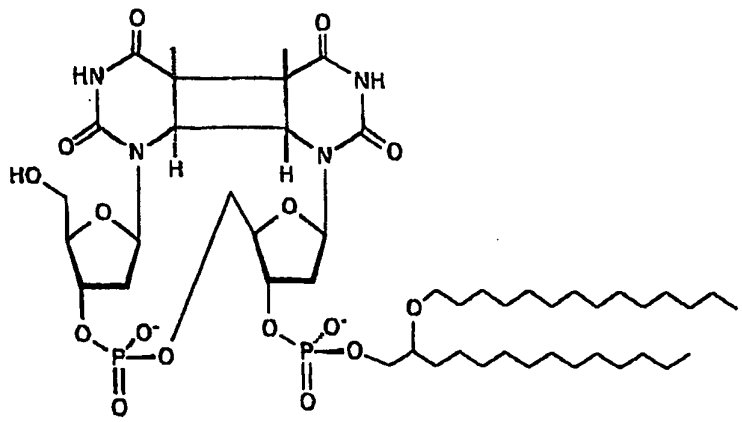
FIG. 19 CpG-containing ODN lipidated at 3'-end

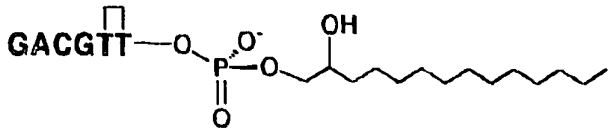


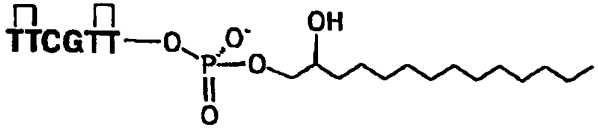
Cyclic molecules have the potential to form stablized three dimensional structures; thus, they are generally better ligands for specefic receptors when the structure of the ligand matches the binding site of the receptor. These structural variations are designed as potential ligands for Toll-like receptors (TLRs).

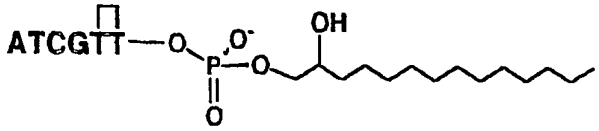
Fig. 20 Lipidated cyclic CpG dinucleotide analogues





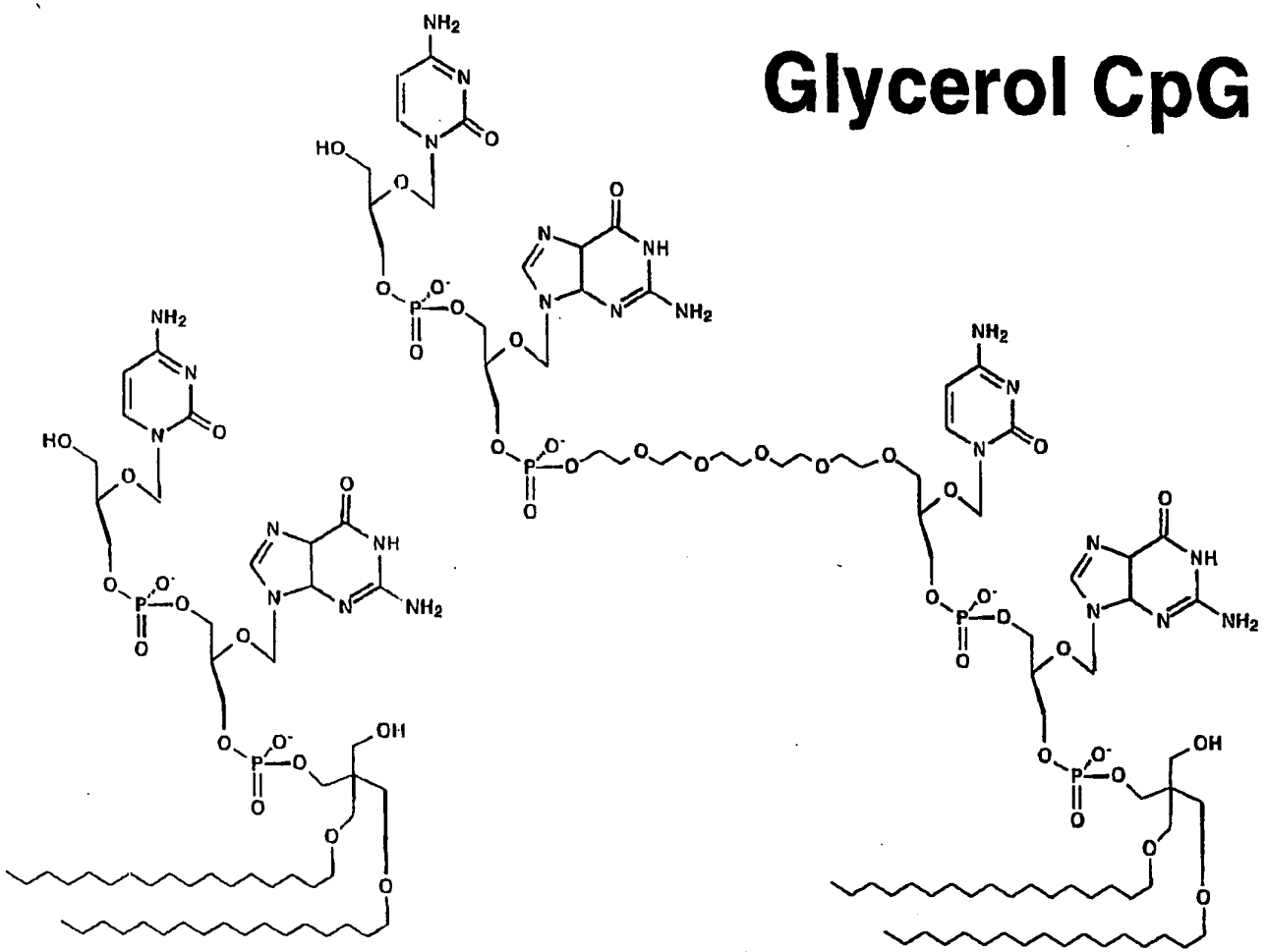






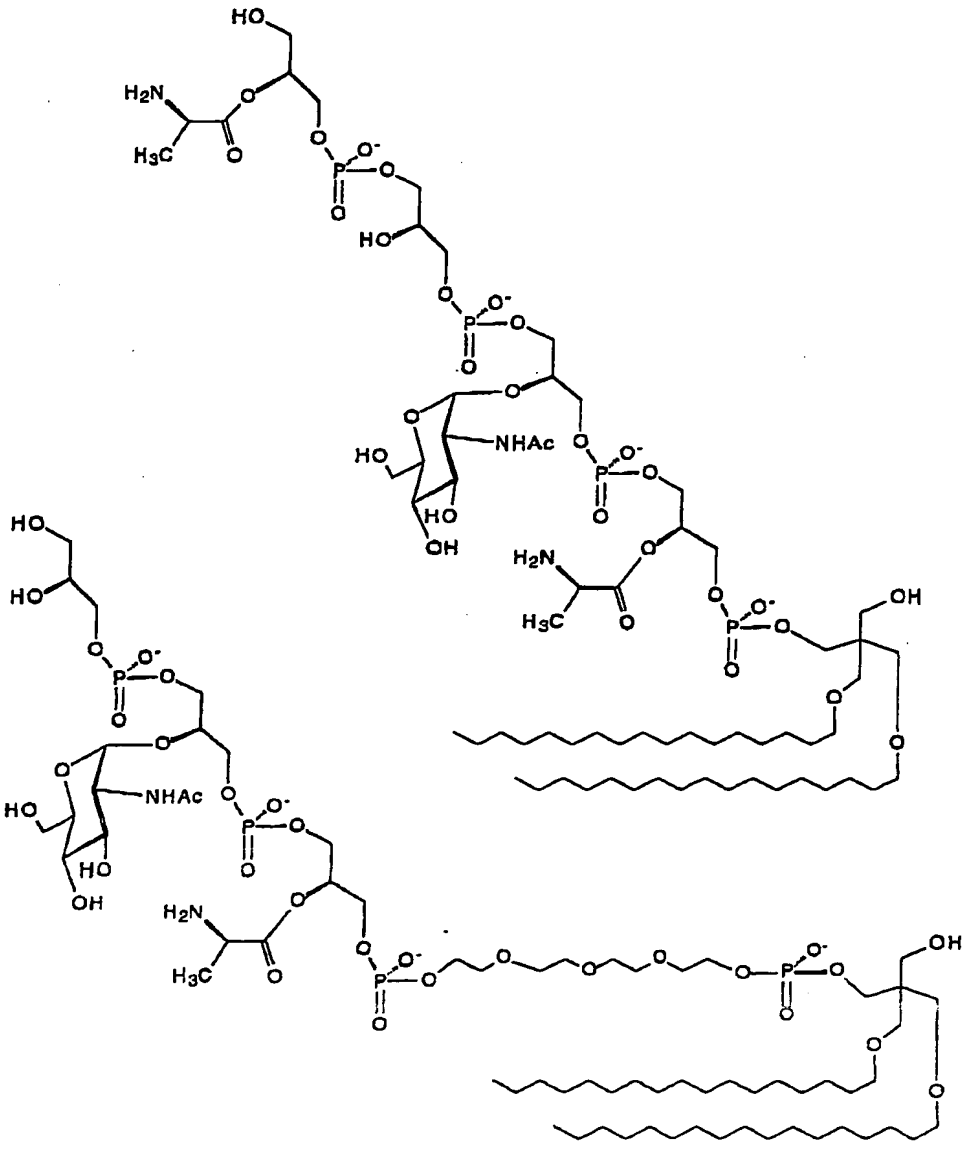
Thymydine dimerization is one of the events behind UV activated DNA lesion. When this occurs, however, human body detects the damage and repair enzymes will be synthesized and the DNA damage will be repaired. It is likely that thymidine dimer may have functioned as a danger signal to which the immune system responses rapidly. Thus, those designed ODN analogues containing thymidine dimer, may have immune modulatory propeties.

FIG. 21 Lipidated ODN containing thymidine dimer



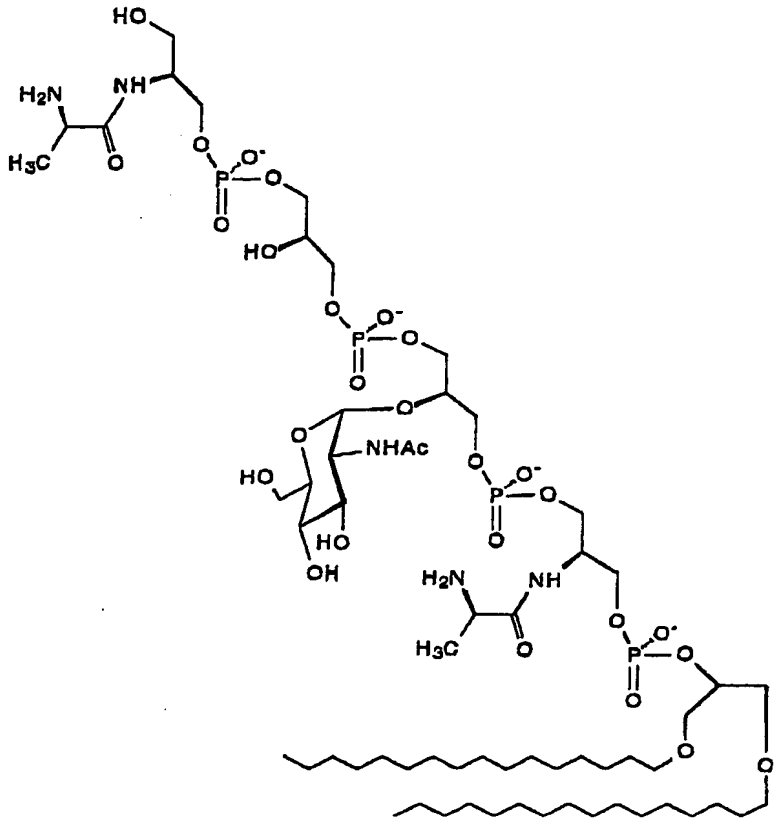
Unmethylated CpG dinucleotide with glycerol backbone: simple GNA derivatives designed as potential immune stimulatory agents.

FIG. 22 Lipidated glycerol CpG analogues



Lipo-teichoic acid (LTA) is the membrane component of Gram-positive bacteria. LTA is well known for its property to activate the innate immune response. Structures derived from LTA are therefore expected to have immune modulatory activities.

FIG. 23 Structures derived from lipo-teichoic acid



Modified Lipo-teichoic acid backbone, wherein the ester linkage between D-Alanine and the secondary hydroxyl group of glycerol unit is replaced by an amide bond, will be more stable toward hydrolytic condition.

FIG. 24 Modified LTA derivatives

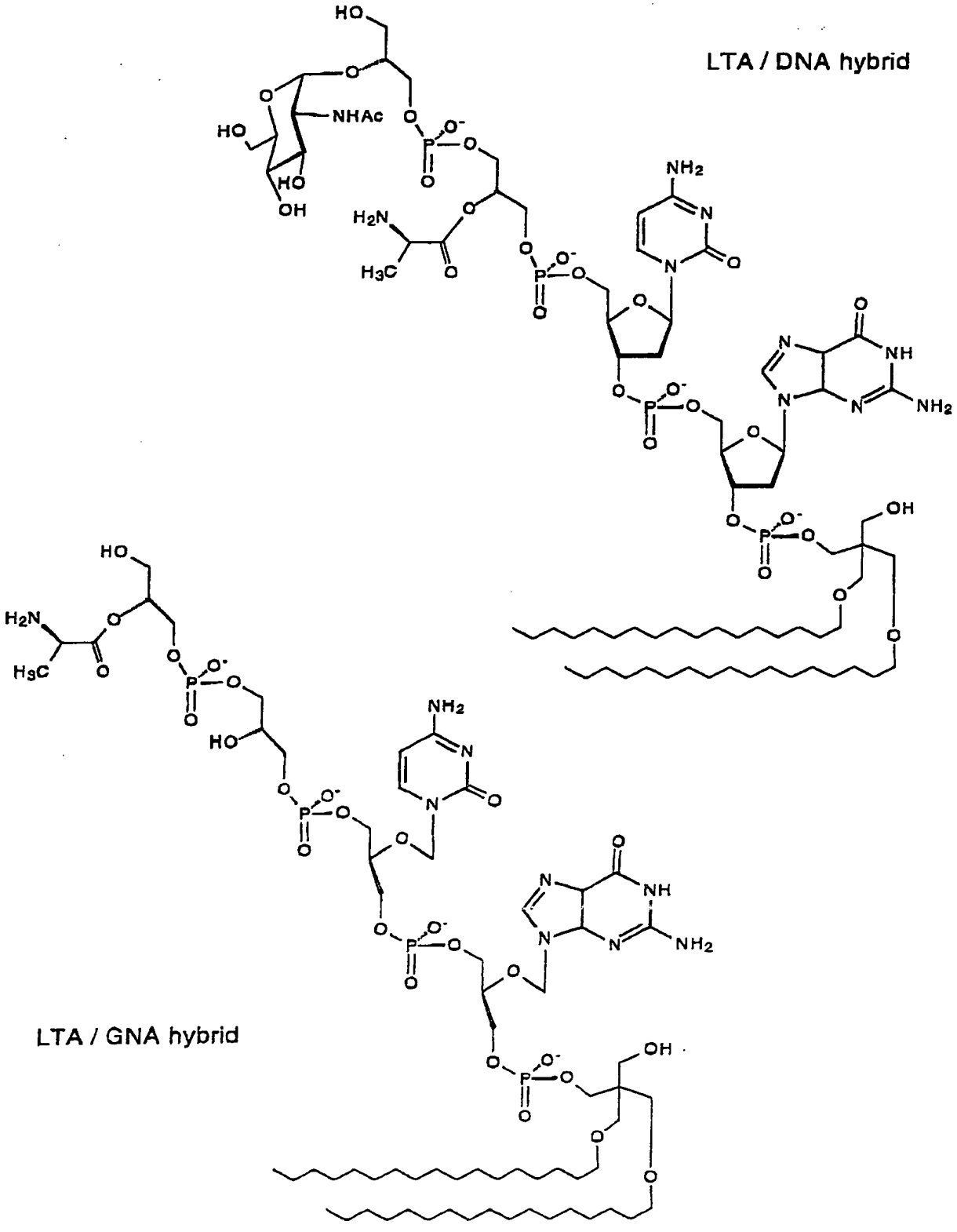


FIG. 25 Hybrid structures derived from LTA, DNA, and GNA

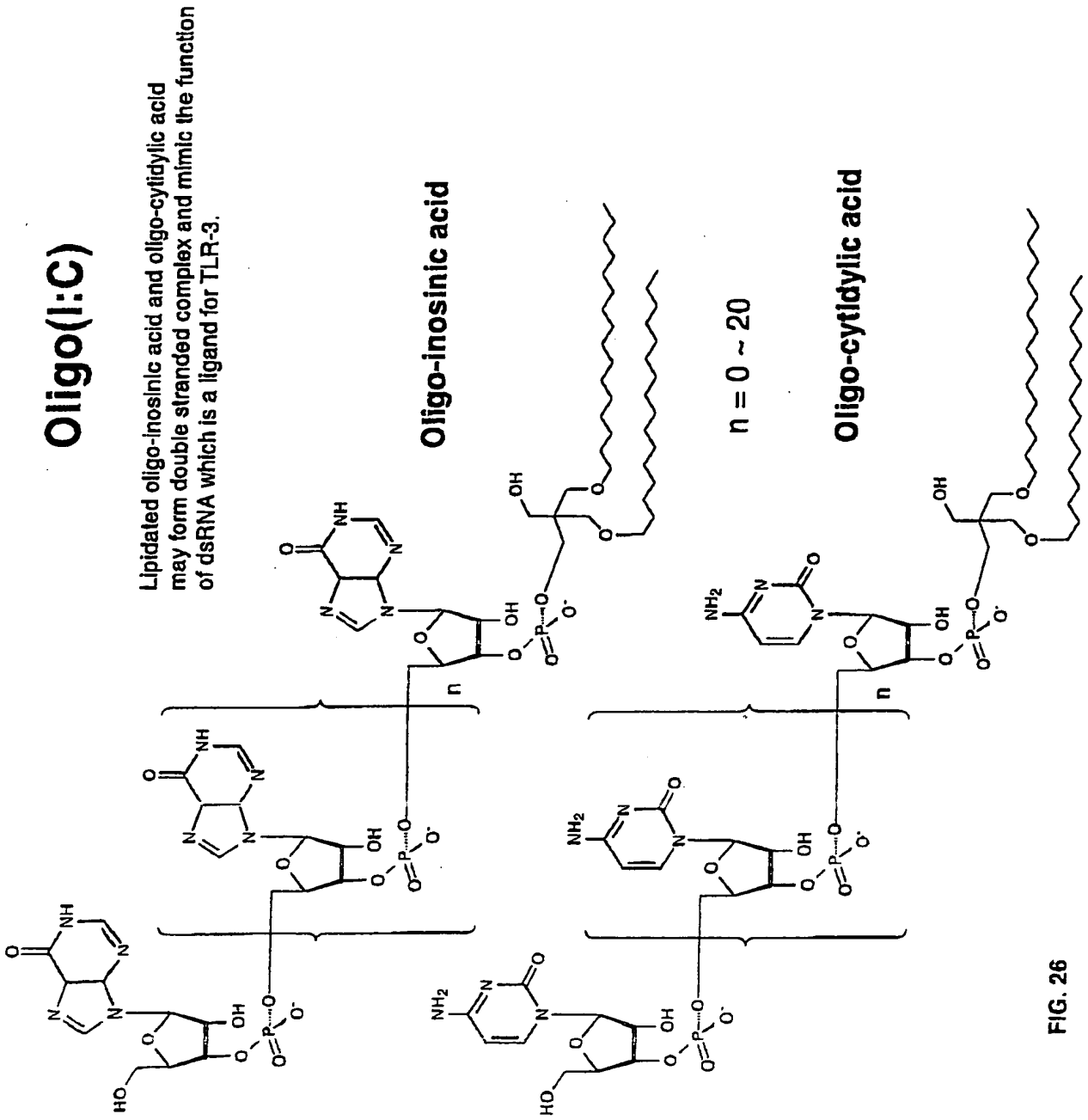

Oligo(I:C)
Lipidated oligo-inosinic acid and oligo-cytidylic acid may form double stranded complex and mimic the function of dsRNA which is a ligand for TLR-3.
Oligo-inosinic acid
n = 0 ~ 20
Oligo-cytidylic acid

FIG. 26

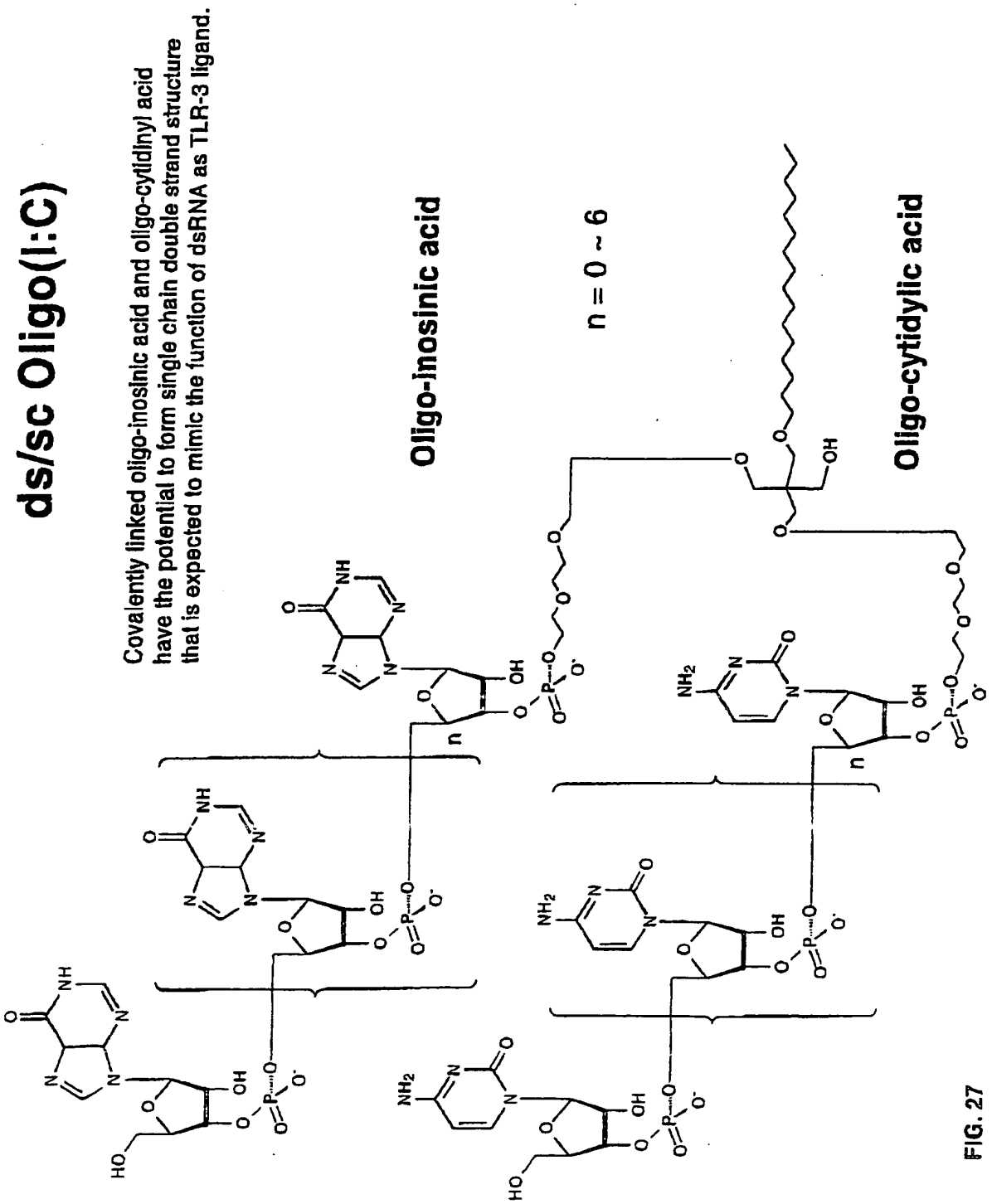

ds/sc Oligo(I:C)
Covalently linked oligo-inosinic acid and oligo-cytidinyl acid have the potential to form single chain double strand structure that is expected to mimic the function of dsRNA as TLR-3 ligand.
Oligo-inosinic acid
n = 0 ~ 6
Oligo-cytidylic acid

FIG. 27

Two covalently linked oligo-inosinic -cytidinyl acid chains also have the potential to form double strand structure that may well mimic the function of dsRNA.

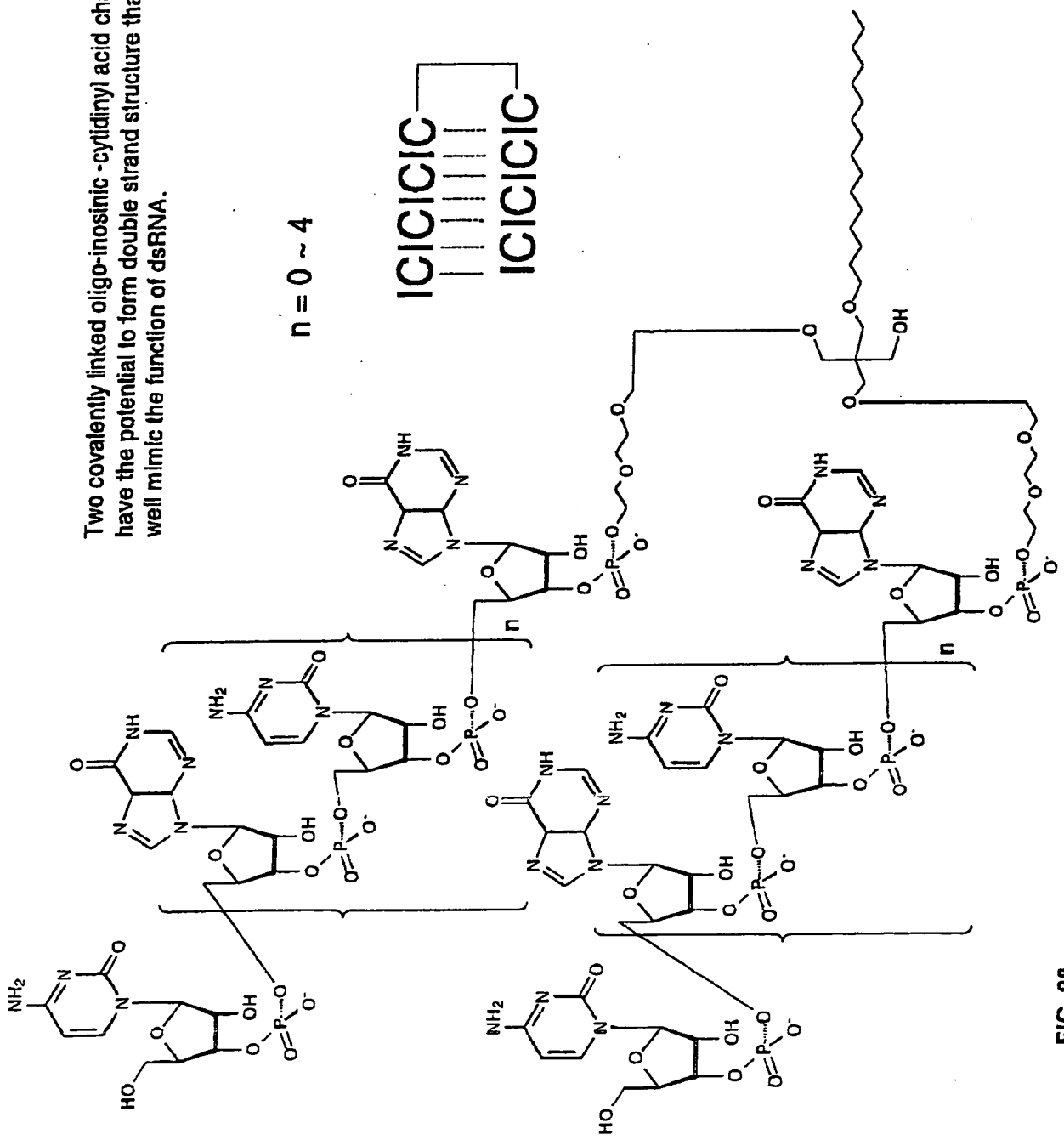


FIG. 28